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# Cryopreservation of collared peccaries (*Tayassu tajacu*) semen using a powdered coconut water (ACP-116c) based extender plus various concentrations of egg yolk and glycerol

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**Abstract**

The objective was to determine the effectiveness of a powdered coconut water-based extender (ACP-116c), plus various concentrations of egg-yolk and glycerol, as an alternative for cryopreservation of collared peccary semen. Twelve ejaculates were obtained from captive adult males by electroejaculation, and evaluated for sperm motility, kinetic rating, viability, morphology, and functional membrane integrity. The ejaculates were apportioned into aliquots that were diluted in Tris plus 10% egg yolk and 3% glycerol, or in ACP-116c plus 10 or 20% egg yolk and 1.5 or 3% glycerol. Samples were frozen in liquid nitrogen and, after 1 mo, thawed at 37 °C for 1 min. After thawing, samples were evaluated as reported for fresh semen, and also for sperm membrane integrity (fluorescent probes) and kinematic parameters (computerized analysis). Results were presented as means ± SEM. Freezing and thawing decreased sperm characteristics relative to fresh semen. Overall, ACP-116c plus 20% egg yolk and 3% glycerol provided better ($P < 0.05$) sperm motility and kinetic rating (48 ± 6.1% and 2.8 ± 0.2, respectively) after thawing than Tris extender (30.4 ± 5.7% and 2.4 ± 0.2). However, there were no differences ($P > 0.05$) among treatments with regard to the other sperm characteristics. Based on computerized motion analysis, total (26.5 ± 5.9%) and progressive (8.1 ± 2.2%) motility were best preserved ($P < 0.05$) with the above-mentioned treatment. In conclusion, a coconut water-based extender, ACP-116c, plus 20% egg yolk and 3% glycerol, was effective for cryopreservation of semen from collared peccaries.



*Keywords:* Tayassu tajacu; Semen; Cryopreservation; Coconut water

## 1. Introduction

For several years, collared peccaries (*Tayassu tajacu*) have been under constant threat of predatory hunting in Latin American countries [1]. Although the International Union for Conservation of Nature (IUCN) considers the populations of these animals as stable [2], they were recently classified as vulnerable to extinction in Brazil, and are in particular considered critically threatened in the Caatinga and Atlantic Forest biomes [3]. Development of protocols for storage of collared peccary semen would allow formation of germ plasm banks and its use in captive breeding programs. Nevertheless, the literature on sperm cryopreservation in this species is still scarce, and only Tris-based extenders have been used [4,5].

The use of coconut water-based extenders has been proposed in the search for alternative semen extenders

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that are non-toxic, buffering, low cost, practical, and effective [6]. Coconut water is a solution composed of salts, proteins, sugars, vitamins, neutral fats, inducers of cell division and various electrolytes, which provides the nutrients necessary for cell preservation [7]. The coconut water extender in powdered form (ACP, ACP Biothecnology, Fortaleza, Brazil) thus emerges as an alternative for use in semen technology, which allows its use even in regions where the fruit of *Cocus nucifera* does not occur naturally [8]. This extender has been effective for conservation of sperm obtained from various species, including the dog [8], fish [9], pig [10], and agouti [11]; however, there is no such report for the collared peccary.

Glycerol is the most common cryoprotectant used for cryopreservation of mammalian semen, including that of the collared peccary [4,5]. The ideal glycerol concentration in the extender represents a balance between its toxic and protecting effects, as high concentrations can reduce sperm fertilizing capacity [12]. In addition, hen's egg yolk has been added to semen extenders for protecting the sperm membrane, by restoring the phospholipids lost during thermal shock that occurs during initial cooling [13]. As it also has buffering capacity, the amount of egg yolk in the media varies according to the buffering capacity of the other components in the extender [14].

The objective of the present study was to determine the effectiveness of a powdered coconut water-based extender (ACP-116c), plus various concentrations of egg-yolk and glycerol, as an alternative for cryopreservation of collared peccary semen.

## 2. Materials and methods

The ethics committee of the UFERSA approved the experimental protocols as well as the animal care procedures used (Process n° 23 091.0253/114). All reagents were obtained from Sigma Aldrich (St. Louis, MO, USA).

### 2.1. Animals

A total of nine sexually mature male collared peccaries, age 40.7 ± 1.6 mo and body weight 22.5 ± 2.8 kg were used. These animals belonged to the Centre of Multiplication of Wild Animals from UFERSA, located in northeast Brazil (Mossoró, RN, Brazil; 5°100' S, 37°100' W). The region is subject to a typical semiarid climate with an average annual temperature of 27 °C. The animals were isolated from the females for 6 mo before commencement of the study and were kept under a 12 h natural photoperiod. Subsequently, they were divided into groups of four and five animals and maintained outdoors in paddocks (3 × 20 m) with a covered area of (3 × 3 m). The animals were fed a diet of sow food and fruits, and water was available *ad libitum.*

### 2.2. Anesthesia

Feed was withheld for 12 h before starting the experiments. They were then physically restrained using a hand net and anesthetized with 5 mg $kg^{-1}$ propofol (Propovan, Cristalia, Fortaleza, Brazil), given iv as a bolus [15]. When the animal showed signs of awakening, additional propofol, approximately 1.25 mg $kg^{-1}$ was given to prolong anesthesia. During the procedure, an indwelling venous catheter was inserted into the cephalic vein for fluid therapy (0.9% physiological saline solution), and physiological parameters were monitored.

### 2.3. Semen collection

The animals were kept in lateral recumbency, and semen was collected using an electroejaculator (Autojac, Neovet, Campinas, SP, Brazil). The electroejaculator probe was 1.3 cm in diameter and 15 cm long (12 cm was inserted into the rectum) [4,5]. The probe had three longitudinal electrodes. The stimulatory cycle included 10 stimuli in each voltage, starting at 5 V, with increases in steps of 1-V up to 12 V. Each electrical stimulus lasted for 3 s, with intermittent breaks of 2 s. The stimulation cycle was maintained for 10 min. Semen was collected in plastic tubes and immediately evaluated.

### 2.4. Semen evaluation

Semen color was noted and volume was measured with micropipettes. Sperm motility and kinetic rating (0–5, with 0 and characterized by no movement and progressive forward movement, respectively) were assessed immediately by evaluating a sample (5 μL) under light microscopy at 100 and 400× magnification. Brome-phenol blue-stained smears [16] were prepared with 5 μL of semen for evaluating sperm viability and morphology, and acrosome integrity using light microscopy (1000×), counting 200 cells per slide. The morphologic defects detected in the sperm were classified as primary or secondary [17]. Following the initial assessment, a 5 μL semen aliquot was diluted in 10% buffered formalin (1 mL) and sperm concentration was determined using a Neubauer counting chamber. The functional integrity of the sperm membrane was evaluated by a hypo-osmotic swelling (HOST) test, using

distilled water (0 mOsm/L) as the hypo-osmotic solution [18].

### 2.5. Extenders and freezing–thawing procedures

A Tris-based extender was used as a control. This extender was comprised of 3.028 g Tris-hydroxymethyl-aminomethane, 1.78 g monohydrated citric acid, and 1.25 g D-fructose dissolved in 100 mL ultrapure water [4]. The extender osmolarity was 295 mOsm/L and the pH was 6.6. Further, 10% *in natura* egg yolk was added to this extender.

The ACP used in the experiment was ACP-116c (ACP Biotecnologia, Fortaleza, Brazil), registered for use in cryopreservation of the collared peccary semen. This product is composed of dehydrated coconut water and pH regulators. A vial of ACP-116c contains 12 g of the product, which must be reconstituted with 50 mL of distilled water. After reconstitution, the extender pH was 7.4 with an osmolarity of 307 mOsm/kg. This extender was tested with the addition of 10% (group ACP10) and 20% (group ACP20) *in natura* egg yolk.

Semen samples were divided into three aliquots immediately after collection and initial evaluation. After dilution in Tris, ACP10 or ACP 20, all samples were stored in a water jacket (30 mL) at 27 °C and equilibrated for 4 h at 5 °C in a refrigerator. After cooling, Tris plus 6% glycerol (also at 5 °C) was slowly added to the control sample, which resulted in a final concentration of 3% glycerol in the extender. Test samples were each divided into two aliquots, to which 3 or 6% glycerol were added (final concentrations of 1.5 and 3% glycerol, respectively). Therefore, after the second dilution, there were four groups: ACP10–1.5, ACP10–3, ACP20–1.5, and ACP20–3. For all samples, the final dilution resulted in a sperm concentration of $100 \times 10^6$ sperm/mL. Sperm motility and kinetic rating were evaluated immediately after addition of glycerol. Samples were packed into 0.25-mL plastic straws and placed horizontally in an insulated box for 20 min, 3 cm above liquid nitrogen ($N_2$). Finally, straws were plunged into liquid $N_2$ [4]. After 1 mo, samples were thawed by immersing the straws in a water bath at 37 °C for 1 min [4]. The semen was immediately evaluated (as reported for fresh semen) and also with computer-assisted semen analysis. In addition, sperm plasma membrane integrity was assessed by fluorescent probes (described below).

### 2.6. Computer-assisted semen analysis

Thawed semen was evaluated by CASA, as described [19]. Semen samples (10 μL) were placed in a Makler chamber, allowed to settle for 1 min and maintained at 37.8 °C. They were then examined in a phase-contrast microcopy system with stroboscopic illumination coupled with a video camera adapted to the Sperm Class Analyzer (SCA Version 3.2.0; Microptic s.l., Barcelona, Spain). The settings of the instrument were adjusted according to boar semen, including temperature, 37 °C; frame rate, 25 frames/sec; minimum contrast, 75; straightness threshold, 45%; low-velocity average pathway (VAP) cut-off, 10; and medium VAP cut-off, 25. Three independent and non-consecutive microscopic fields were randomly selected and scanned. The following endpoints were analyzed: number of counted cells, total motility (%), progressive motility (%), VAP (mm/sec), velocity straight line (VSL; mm/sec), curvilinear velocity (VCL; mm/sec), amplitude of lateral head (ALH; mm), beat cross frequency (BCF; Hz), straightness (STR; %), and linearity (LIN; %). According to the low VAP cut-off (LVV) point and the medium VAP cut-off (MVV) point, the overall sperm population was subdivided into four categories: rapid, with VAP > MVV; medium, with (LVV < VAP < MVV); slow, with VAP < LVV; and static, the proportion of cells not moving.

### 2.7. Evaluation of post-thaw sperm plasma membrane integrity

Plasma membrane integrity was assessed with a fluorescent solution containing fluorophores 6-carboxyfluorescein diacetate (0.46 mg C-FDA/1 mL dimethylsulfoxide) and propidium iodide (0.5 mg PI/1 mL 0.9% saline solution). Initially, fresh ejaculates were used for standardizing protocols for the evaluation of collared peccary sperm. One aliquot of 10 μL thawed semen was extended in 40 μL fluorescent solution. After 10 min, slides of the stained samples were evaluated by epifluorescence microscopy (Episcopic Fluorescent attachment "EFA" Halogen Lamp Set, Leica, Kista, Sweden). For each sample stained with CFDA/PI, 200 sperm were counted and classified as having or not having an intact plasmalemma (based on color). Cell membranes stained in green (CFDA) were considered intact, whereas those stained in red (PI) or partially stained were considered non-intact [20].

### 2.8. Statistical analyses

A total of 12 replicates were performed for each treatment. The results were expressed as mean ± SEM. Data were checked for normality by the Shapiro–Wilk test, and for homoscedasticity by Levene's test using the StatView 5.0 (SAS Institute, Inc, Cary, NY, USA). Values expressed in percentages were *arcsine* trans-

Table 1
Sperm characteristics in fresh ejaculates of collared peccaries (*Tayassu tajacu*) collected by electroejaculation (n = 7 males; 12 ejaculates).

| | Mean ± SEM | Range |
|---|---|---|
| Volume (mL) | 2.8 ± 0.7 | 0.6–7.5 |
| Sperm concentration (x10[6]) | 765 ± 313.7 | 150–4130 |
| Sperm motility (%) | 86.7 ± 2.6 | 5.0–95 |
| Kinetic rating (0–5) | 4.4 ± 0.3 | 3–5 |
| Sperm viability (%) | 92.3 ± 1.6 | 83–100 |
| Hypo-osmotic swelling test (%) | 75.3 ± 2.3 | 65.0–86 |
| Sperm morphology | | |
| Normal (%) | 83.2 ± 2.2 | 67–92 |
| Primary defects (%) | 0.7 ± 0.4 | 0–3 |
| Secondary defects (%) | 16.7 ± 2.3 | 8–33 |
| Total defects (%) | 16.8 ± 2.2 | 8–33 |

formed before analysis. Comparisons among various extenders on the seminal parameters were made by ANOVA followed by the Student Newman Keul's test. The effect of the extenders on sperm vigor was evaluated by the nonparametric Mann-Whitney test. Differences were considered significant when $P < 0.05$.

## 3. Results

### 3.1. Fresh and extended semen

A total of 20 attempts for semen collection were conducted in nine animals. Successful ejaculation was achieved only in seven animals, from which 17 ejaculates were obtained. From those ejaculates, only 12 were used; the other five ejaculates were excluded, because of low volume, motility, or vigor. Regarding the ejaculates used, two ejaculates were collected from each of three animals, three other males ejaculated only once, and three samples were collected from one male. The twelve ejaculates used were white and watery, with an average volume of 2.8 ± 0.7 mL. The other semen characteristics are shown (Table 1). The addition of the extenders induced no decline ($P > 0.05$) in sperm motility or kinetic rating in any group. However, the addition of glycerol significantly ($P < 0.05$) reduced sperm motility and kinetic rating in all samples.

### 3.2. Post-thaw sperm characteristics

The freezing and thawing procedures induced a decrease in the sperm characteristics relative to fresh semen (Table 2). Regarding comparisons among groups, ACP-116c plus 20% egg yolk and 3% glycerol provided better results for sperm motility and kinetic rating after thawing than Tris extender ($P < 0.05$). However, none of the other sperm characteristics differed ($P > 0.05$) among treatments.

In view of the kinematic parameters generated by CASA (Table 3), total and progressive motility were better preserved in the use of ACP-16c plus 20% egg yolk and 1.5 or 3% glycerol ($P < 0.05$). The same groups provided the best results for STR and LIN ($P < 0.05$).

## 4. Discussion

In the present research, all samples (regardless of the extender) had reduced semen endpoints after thawing. In that regard, it is well known that sperm undergo successive and opposing changes in cell volume by osmotic changes caused during glycerol addition, freez-

Table 2
Characteristics of frozen-thawed sperm from collared peccaries (n = 12 ejaculates), extended in Tris (control group) or powdered coconut water (ACP-116c) plus various concentrations of EY and glycerol (GLY).

| | Tris plus 10% EY and 3% GLY | ACP plus 10% EY and 1.5% GLY | ACP plus 10% EY and 3% GLY | ACP plus 20% EY and 1.5% GLY | ACP plus 20% EY and 3% GLY |
|---|---|---|---|---|---|
| Sperm motility (%) | 30.4 ± 5.7[a] | 33.4 ± 5.5[ab] | 40.8 ± 6.9[ab] | 44.6 ± 6.2[ab] | 48.3 ± 6.1[b] |
| Kinetic rating (0–5) | 2.4 ± 0.2[a] | 3.0 ± 0.2[b] | 2.9 ± 0.2[b] | 3.0 ± 0.2[b] | 2.8 ± 0.2[b] |
| Sperm viability (%) | 36.6 ± 5.6 | 42.7 ± 4.8 | 40.4 ± 5.3 | 41.7 ± 4 | 45.3 ± 4.8 |
| Hypo-osmotic swelling test (%) | 50 ± 5.8 | 50.3 ± 6.5 | 61.2 ± 5.6 | 57.5 ± 5.7 | 59.9 ± 5 |
| Plasma membrane integrity (%) | 34.1 ± 7.1 | 30 ± 6.8 | 33.3 ± 7.9 | 33.4 ± 6.5 | 25.8 ± 5 |
| Sperm morphology | | | | | |
| Normal (%) | 64.6 ± 5.4 | 63.4 ± 6.0 | 65.8 ± 5.3 | 57.5 ± 5.7 | 64.8 ± 5.6 |
| Primary defects (%) | 3.1 ± 1.1 | 0.9 ± 0.5 | 6.8 ± 3.93 | 2.3 ± 0.8 | 3.3 ± 0.9 |
| Secondary defects (%) | 33.2 ± 5.6 | 35.7 ± 6.0 | 27.3 ± 5.8 | 36.9 ± 6.9 | 32 ± 5.8 |
| Total defects (%) | 36.3 ± 5.3 | 36.6 ± 6.0 | 34.1 ± 5.3 | 30.6 ± 3.92 | 35.3 ± 5.6 |

[a,b] Within a row, means without a common superscript differ ($P < 0.05$).

Table 3
Motility endpoints, measured by computer-assisted semen analysis, of frozen-thawed sperm from collared peccaries (n = 12 ejaculates), extended in Tris (control group) or powdered coconut water (ACP-116c) plus various concentrations of EY and glycerol (GLY).

| Endpoint | Tris plus 10% EY and 3% GLY | ACP plus 10% EY and 1.5% GLY | ACP plus 10% EY and 3% GLY | ACP plus 20% EY and 1.5% GLY | ACP plus 20% EY and 3% GLY |
|---|---|---|---|---|---|
| Total motility (%) | 13.1 ± 3$^{ab}$ | 8.6 ± 2.5$^{bc}$ | 6.6 ± 2.1$^{a}$ | 23.6 ± 4.9$^{b}$ | 26.5 ± 5.9$^{b}$ |
| Progressive motility (%) | 3.7 ± 1.1$^{ab}$ | 2 ± 0.7$^{b}$ | 1.8 ± 0.5$^{b}$ | 6.1 ± 1.7$^{b}$ | 8.1 ± 2.2$^{b}$ |
| Velocity curvilinear – VCL ($\mu$m/sec) | 26.1 ± 2.1$^{a}$ | 21.1 ± 4.2$^{a}$ | 31 ± 4.4$^{a}$ | 25.2 ± 2.3$^{a}$ | 25.1 ± 1.2$^{a}$ |
| Velocity straight line – VSL ($\mu$m/sec) | 8.4 ± 0.7$^{b}$ | 8.9 ± 1.8$^{ab}$ | 12.5 ± 1.7$^{b}$ | 10 ± 0.9$^{ab}$ | 10.2 ± 0.5$^{ab}$ |
| Velocity average pathway – VAP ($\mu$m/sec) | 13.7 ± 1.1$^{a}$ | 13.1 ± 2.5$^{a}$ | 18 ± 2.2$^{a}$ | 15.1 ± 1.3$^{a}$ | 15.3 ± 0.6$^{a}$ |
| Linearity – LIN (%) | 32.3 ± 1.3$^{b}$ | 32.2 ± 5.9$^{ab}$ | 37.7 ± 4.1$^{ab}$ | 39.9 ± 2.3$^{b}$ | 41.2 ± 1.7$^{b}$ |
| Straightness – STR (%) | 61.1 ± 1.9$^{b}$ | 50.3 ± 8.9$^{ab}$ | 63.4 ± 6.1$^{ab}$ | 66.7 ± 2.1$^{ab}$ | 67.1 ± 1.7$^{a}$ |
| Amplitude lateral head – ALH ($\mu$m) | 1.9 ± 0.3$^{a}$ | 1.7 ± 0.4$^{a}$ | 2.1 ± 0.2$^{a}$ | 2.2 ± 0.2$^{a}$ | 2.3 ± 0.1$^{a}$ |
| Beat cross frequency – BCF (Hz) | 3.3 ± 0.8$^{a}$ | 3.5 ± 1.0$^{a}$ | 5.2 ± 0.8$^{a}$ | 4.5 ± 0.7$^{a}$ | 4.1 ± 0.7$^{a}$ |

$^{a,b,c}$ Within a row, means without a common superscript differed ($P < 0.05$).

ing, and thawing [21]. Moreover, the fluidity and functional properties of the sperm membranes are modified by temperature changes during the cooling and freezing phases, as well as during thawing [22]. This results in a reduction of their selective permeability, which ultimately leads to a major influx of calcium [23]. The plasma membrane is the primary site of injury for cryopreserved sperm and the main damage occurs during freezing and thawing [24], resulting in a substantial loss of viability.

Results obtained with Tris-based extender after thawing seemed similar to those previously reported [4,5]. However, it was also demonstrated that ACP-116c could be efficiently used as an alternative for semen freezing in collared peccaries. Also, the use of ACP-116c plus 20% egg yolk and 3% glycerol yielded significantly better preservation of the sperm motility and kinetic rating when compared to Tris. The biochemical characteristics of the ACP are very similar to those of fresh coconut water. In addition to its rich composition [7], coconut water has arginine, lysine, and high concentrations of $Na^+$ and $K^+$, which were important factors for reversible suppression of sperm motility and longevity in the honey bee [25]. Furthermore, it was previously demonstrated that indole-3-acetic acid (IAA), a plant hormone present in coconut water, could enhance conservation of sperm of swine [26], a domestic species closely related to the collared peccary [27].

Based on computerized analysis, 20% was the optimal concentration of egg yolk to be added to ACP-116c for collared peccary semen cryopreservation. These results seemed similar to those reported for domestic swine in which the extenders used for the sperm cryopreservation usually contain 20% egg yolk [28,29]. There were no significant differences among treatments with regard to other sperm characteristics. It is reported that high egg yolk concentrations do not interfere with sperm vitality and morphology [30].

Glycerol is the main cryoprotectant used for freezing semen from various species; concentrations below 2% or above 6% are reported to be deleterious, because of low cryoprotectant activity or sperm toxicity, respectively [31]. In the present study, progressive motility was decreased when 1.5% glycerol was added to ACP-116c for collared peccary semen. These results seemed different from those reported for domestic swine, in which glycerol concentrations varying from 1 to 3% are usual for maximum post-thaw survival. It is noteworthy that the primary cryoprotective effect of glycerol on swine sperm might be extracellular [32].

It is proposed that glycerol, as well as other penetrating cryoprotectants, are osmotically active; they change the water content and cause stress while entering or exiting the cell [33]. In the specific case of pig sperm, these cells use glycerol as a carbon source, even in the presence of other substrates, such as glucose [34]. Beside, extenders with glycerol addition to the egg yolk have a synergistic effect, increasing the post-thaw survival of sperm cells [35]. The greater sperm survival of the samples frozen in extenders can thus be attributed to a reduction in osmotic stress, owing to the combination of cryoprotectants used in the extender.

In general, the use of additives improves the quality of preserved sperm. It is speculated that the benefit of this association might be attributed to the different nature of the molecules involved in their effect. Egg yolk improves sperm viability during storage [36]. The addition of glycerol to extenders containing egg yolk has a synergistic protective effect during cooling and freezing [37]. Cryoprotectant concentrations for the freezing of semen should be chosen such that the osmotic tolerance limits of the cells are not exceeded. Both the loss in cell viability because of exposure to cryoprotectants as well as cell survival after freeze–thaw and return to isosmotic conditions, should be considered while designing cryopreservation protocol [38].

Egg yolk was not totally dissolved when ACP was used, thereby increasing the amount of debris that can interfere with computerized analysis of semen, as previously reported for dogs [8]. This was attributed to the egg yolk, which was used *in natura*, without any centrifugation. Therefore, inclusion of egg yolk low-density lipoprotein (LDL) [39] and sodium dodecyl-sulfate-based detergents [40] have been suggested for collared peccary semen cryopreservation.

In conclusion, the present research attempted to improve semen freezing protocols to enable formation of a germ plasm bank for collared peccaries. In this study, a coconut water-based extender, ACP-116c, was effective for cryopreservation of semen of this species. The addition of 20% egg yolk and 3% glycerol to this extender is recommended to achieve satisfactory post-thaw sperm quality.

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## References

[1] Bodmer RE, Aquino R, Puertas P, Reyes C, Fang T, Gottdenker N. Manejo y uso sustentable de Pecaríes en la Amazonía Peruana [Sustainable management and use of peccaries in the Peruvian Amazon]. Occasional Paper of the IUCN species survival Commission Number 18; 1997. IUCN-Sur, Quito, Ecuador.

[2] IUCN. The IUCN red list of threatened species. Available at http://www.iucnredlist.org. Accessed December 06, 2011.

[3] Chico I-I. Mendes de Conservação da Biodiversidade: six species of ungulate mammals are threatened for extinction in Brazil. Available at http://www.icmbio.org.br. Accessed February 22, 2011.

[4] Castelo TS, Bezerra FS, Souza AL, Moreira MA, Paula VV, Oliveira MF, et al. Influence of the thawing rate on the cryopreservation of semen from collared peccaries (Tayassu tajacu) using Tris-based extenders. Theriogenology 2010;74:1060–5.

[5] Castelo TS, Bezerra FS, Lima GL, Alves HM, Oliveira IR, Santos EA, et al. Effect of centrifugation and sugar supplementation on the semen cryopreservation of captive collared peccaries (*Tayassu tajacu*). Cryobiology 2010;61:275–9.

[6] Cardoso RCS, Silva AR, Silva LDM. Use of the powdered coconut water (ACP-106®) for cryopreservation of canine spermatozoa. Anim Reprod 2005;2:257–62.

[7] Blume H, Marques AP Jr. Avaliação da água de coco no cultivo e criopreservação de embriões murídeos [Evaluation of coconut water for the culture and cryopreservation of murid embryos]. Rev Bras Reprod Anim 1994;18:97–104.

[8] Cardoso RC, Silva AR, Silva LD, Chirinéa VH, Souza FF, Lopes MD. Evaluation of fertilizing potential of frozen-thawed dog spermatozoa diluted in ACP-106 using an in vitro sperm-oocyte interaction assay. Reprod Domest Anim 2007;42:11–6.

[9] Viveiros ATM, Maria AN, Orfão LH, Carvalho MAM, Nunes JF. Powder coconut water (ACP®) as extender for semen cryopreservation of Brazilian migratory fish species. International Symposium on Reproductive Physiology of Fish 2007. Saint Malo, France, 2007, pp. 232.

[10] Toniolli R, Toniollo GH, Franceschini PH, Fmac M. Use of powder coconut water as extender (ACP-103®) for boar semen longer preservation: in vitro and in vivo evaluations. Braz J Vet Res Anim Sci 2010;62:1072–9.

[11] Silva MA, Peixoto GC, Santos EA, Castelo TS, Oliveira MF, Silva AR. Recovery and cryopreservation of epididymal sperm from agouti (Dasiprocta aguti) using powdered coconut water (ACP-109c) and Tris extenders. Theriogenology 2011;76:1084–9.

[12] England GCW. Cryopreservation of dog semen: a review. J Reprod Fert 1993;47:243–55.

[13] Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. J Androl 1990;11:73–88.

[14] Farstad W. Semen cryopreservation in dogs and foxes. Anim Reprod Sci 1996;42:251–60.

[15] Souza AL, Castelo TS, Queiroz JP, Barros IO, Paula VV, Oliveira MF, et al. Evaluation of anesthetic protocol for the collection of semen from captive collared peccaries (Tayassu tajacu) by electroejaculation. Anim Reprod Sci 2009;116:370–5.

[16] Derivaux J. Reprodução dos animais domésticos [Reproduction of domestic animals]. Zaragoza: Ed. Acribia. 1980, pp. 446.

[17] Blom E. Interpretation of spermatic cytology of bulls. Fert Steril 1950;1:223–38.

[18] Moussa M, Marinet V, Trimeche A, Tainturier D, Anton M. Low density lipoproteins extract from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. Theriogenology 2002;57:1695–706.

[19] Verstegen J, Iguer-Ouada M, Onclin K. Computer assisted analyzers in andrology research and veterinary practice. Theriogenology 2002;57:149–79.

[20] Silva AR, Cardoso RdeC, Chirinéa VH, Lopes MD, Souza FF. Prognostic value of canine frozen thawed semen parameters on in vitro sperm oocyte interactions. Theriogenology 2006;66:456–62.
[21] Mann T. Biochemistry of semen. In: Greep RO, Astwood EB, editors. Handbook of physiology. Washington DC: American Physiology Society; 1975;5:321–47.
[22] Holt WV. Basic aspects of frozen storage of semen. Anim Reprod Sci 2000;62:3–22.
[23] Maxwell WM, Johnson LA. Chlortetracycline analysis of boar spermatozoa after incubation, flow cytometric sorting, cooling or cryopreservation. Mol Reprod Dev 1997;46:408–18.
[24] Bailey JL, Morrier A, Cormier N. Semen cryopreservation in farm species: an update. Can J Anim Sci 2003;83:393–401.
[25] Almeida R, Soares AEE. Usage of green coconut water and different tissue culture media for in vitro honey bee semen storage (*Apis melifera*; Himenoptera: Apoidea). Interciencia 2002;27:317–21.
[26] Toniolli R, Bussière J, Courot M, Magistrini M, Combarnous Y. Effect of indole-3-acetic acid (plant auxin) on the preservation at 15 degrees C of boar semen for artificial insemination. Reprod Nutr Dev 1996;36:503–11.
[27] Cavalcante-Filho MF, Miglino MA, Machado GV, Bevilacqua EMAF, Neves WC. Comparative study of the morphology of the stomach of White lipped peccary (*Tayassu pecari*) and of the collared peccary (*Tayassu tajacu*). Braz J Morph Sci 1998;15:206–7.
[28] Garcia JC, Dominguez JC, Pena FJ, Alegre B, Gonzalez R, Castro MJ, et al. Thawing boar semen in the presence of seminal plasma: effects on sperm quality and fertility. Anim Reprod Sci 2010;119:160–5.
[29] Spencer KW, Purdy PH, Blackburn HD, Spiller SF, Stewart TS, Knox RV. Effect of number of motile, frozen-thawed boar sperm and number of fixed-time inseminations on fertility in estrous-synchronized gilts. Anim Reprod Sci 2010;121:259–66.
[30] Wall RJ, Foote RH. Fertility of bull semen frozen and store in clarified egg yolk-Tris-glycerol extender. J Dairy Sci 1999;82:817–21.
[31] Fahy GM. The relevance of cryoprotectant "toxicity" to cryobiology. Cryobiology 1986;23:1–13.
[32] Paquignon M. Freezing and thawing extenders of boar spermatozoa. In: L.A. Johnson and K. Larsson, editors. Deep Freezing of Boar Semen. Sweden: Swedish Univ of Agric Sci, Uppsala, 1985, pp. 129–46.
[33] Bhur MM, Fiser P, Bailey JL, Curtis EF. Cryopreservation in different concentrations of glycerol alters boar sperm and their membranes. J Androl 2001;22:961–9.
[34] Óshe T, Dacheaux JL, Paquignon M. Metabolism of boar spermatozoa before, during preparation for, and after storage in liquid nitrogen. J Reprod Fert 1979;55:277–85.
[35] Paz P, Alvarez-Rodriguez M, Nicolas M, Alvarez M, Chamorro MA. Borraga′ n S, Martinez-pastor F, Anel L. Optimization of glycerol concentration and freezing rate in the cryopreservation of ejaculate from brown bear (*Ursus arctos*). Reprod Domest Anim 2012;47:105–12.
[36] Salamon S, Maxwell WM. Storage of ram semen. Anim Reprod Sci 2000;62:77–111.
[37] Pace MM, Graham EF. Components in egg yolk which protect bovine sperm during freezing. J Anim Sci 1974;39:1144–9.
[38] Hoffmann N, Oldenhof H, Morandini C, Rohn K, Sieme H. Optimal concentrations of cryoprotective agents for semen from stallions that are classified "good" or "poor" for freezing. Anim Reprod Sci 2011;125:112–8.
[39] Bencharif D, Amirat L, Anton M, Schmitt E, Desherces S, Delhomme G, et al. The advantages of LDL (low density lipoproteins) in the cryopreservation of canine semen. Theriogenology 2008;70:1478–88.
[40] Morton KM, Evans G, Maxwell WM. Effect of glycerol concentration, Equex STM supplementation and liquid storage prior to freezing on the motility and acrosome integrity of frozen-thawed epididymal alpaca (Vicugna pacos) sperm. Theriogenology 2010;74:311–6.